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Nitrosamine Precursors in the Adipose Tissue of Pork Bellies

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ABSTRACT

Adipose tissue has been implicated as the primary site of nitrosopyrrolidine (NPYR) production in fried bacon. In a study on the precursors of NPYR, pork belly adipose tissue was extracted with $CHCl_3$/MeOH and yielded an organic layer, an aqueous polar fraction, and a tissue residue. Fractions were heated with nitrite, and the polar fraction accounted for most of the nitrosamines in intact adipose tissue. Fractionation of the polar fraction yielded an eluate that was collected in seven aliquots. All aliquots formed N-nitrosodimethylamine when heated with nitrite, but the bulk of NPYR was formed from an eluate corresponding to the first of three UV absorbent bands at 254 nm.

INTRODUCTION

OF ALL THE CURED MEAT products tested for nitrosamines (NAs), bacon presents the greatest concern to humans because of the consistent presence of N-nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR) generated when it is fried. Nitrosamines are formed by the reaction of $NaNO_2$ with secondary amines that are either present or formed in the product. Most of this class of compounds have been found to be carcinogenic in animal tests (Magee, 1971). The Food Safety and Quality Service (FSQS), USDA, has therefore promulgated regulations which restrict the addition of $NaNO_2$ to 120 ppm and require the addition of 550 ppm of sodium erythorbate or sodium ascorbate to pork belly cure solutions to reduce the concentration of NPYR, the principal NA formed. In addition, FSQS has set a violative level of 10 ppb and an action level of 17 ppb for NPYR in fried bacon.

Potential precursors for NPYR have been investigated in model systems, with proline, hydroxyproline, putrescine, spermidine, and glycine used as standards (Bills et al., 1973; Huxel et al., 1974; Enders and Ceh, 1971). These studies, although valid for model systems, may not reflect the reactions occurring in a real meat matrix. The concentration of proline has been determined in fried bacon (Spinelli-Gugger et al., 1980; Mottram et al., 1977) and in collagen (Patterson et al., 1976) because this amino acid is considered to be the principal precursor to NPYR. Gray and Collins (1978) and Nakamura et al. (1976), using an excess of proline and nitrite, determined the optimum temperature for NPYR formation in bacon. Fiddler et al. (1974), Patterson et al. (1976), and Coleman (1978) indicated that NPYR formation occurred primarily in the adipose tissue. There has been, however, no systematic study of the components present in the adipose tissue that could serve as the NA presursor. This paper reports the results of such a study.

EXPERIMENTAL

Sample preparation

Pork bellies were obtained within 24 hr after slaughter. The adipose tissue was manually separated from the lean tissue based on visual observation. The adipose tissue was then ground and thoroughly mixed three times to insure sample homogeneity, then stored at −20°C until use.

Isolation procedure

An outline of the extraction is shown in Figure 1. Specifically, 100g of the pork belly adipose tissue was blended with 100 ml Folch's reagent ($CHCl_3$-MeOH, 2:1) for 3 min in a Waring Blendor at maximum speed. The resulting slurry was centrifuged and the supernatant liquid was separated into an aqueous polar layer (fraction I), an organic nonpolar layer (fraction II), and a tissue residue (residue III) which was trapped between the two liquid phases. The tissue residue was reextracted two additional times with Folch's reagent, and similar phases were combined. The remaining tissue residue was slurried in a blendor three times with 100 ml H_2O and centrifuged, and the supernatants were combined. The organic layer II was washed three times with 100 ml H_2O and allowed to separate. All aqueous extracts were combined with lyophilized to yield polar fraction I. The organic layer II was reduced to dryness on a rotary evaporator to give the nonpolar fraction II.

Column chromatographic separation

Gel filtration chromatography was used to further fractionate the polar fraction I. Optimal separation was achieved with a 50 cm x 2.5 cm column packed with Sephadex G-15 with an eluant of 0.05 M ammonium acetate at a flow rate of 0.5 ml/min. The void volume as determined by application of Blue Dextran 2000 was discarded, and 80 fractions of 5-ml were collected by use of a Gilson fraction collector. All chromatographic separations were cariried out in a cold room at 5°C. The eluant was either continuously monitored as it eluted from the column with a Waters fixed wavelength monitor (254 nm) or each fraction was individually scanned (190–390 nm) with a Perkin-Elmer 202 spectrophotometer.

HPLC of fraction I

Several of the UV absorbing fractions isolated by gel filtration chromatography were rechromatographed with a Waters liquid chromatograph (Series 6000) equipped with a 254 nm detector. Separation was effected with 10μ Bondapak C_{18} column (3.9 mm x 30 cm) and a mobile phase of a 10 mM KH_2PO_4 solution at a flow rate of 0.5 ml/min.

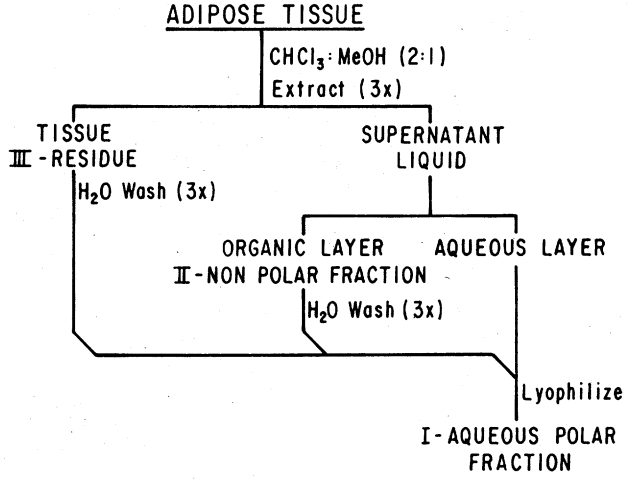

Fig. 1—Flow chart of procedure for extraction of adipose tissue.

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Nitrosamine formation

To aliquots from each of the three primary fractions I, II, and III and subfractions from fraction I, 50 mg $NaNO_2$ were added. To facilitate frying, 25g of hydrogenated oil was added to all fractions except II, which was lipid in nature. Each sample was heated at 170°C (Pensabene et al., 1974) for 4 min in a beaker immersed in a silicone oil bath to simulate conditions of frying bacon. The reaction mixture was stirred continuously to reduce bumping, and the beaker was covered to minimize the loss of volatile NAs.

Nitrosamine analysis

The samples were analyzed for volatile NAs by the isolation procedure of White et al. (1974) and detected and quantitated by gas liquid chromatography (GLC) interfaced with a Thermal Energy Analyzer (Model 502) under chromatographic and instrumental conditions previously described by Pensabene et al. (1979). Mass spectral confirmation was carried out with a Varian-Aerograph 2700 gas chromatograph, equipped with a 6 ft x 1/4 in. (o.d.) glass column packed with 15% Carbowax 20M-TPA, and connected to a Varian MAT 311A mass spectrometer (Lakritz and Kimoto, 1980). In this investigation, additional sample clean up with Silica Gel and acidified Florasil was required (Fazio et al., 1972) after the initial isolation.

RESULTS & DISCUSSION

THE CONCENTRATIONS of NDMA (Table 1) and NPYR (Table 2) were determined in the intact adipose tissue, polar, nonpolar, and residue fractions obtained from 11 samples, based on the weight of adipose tissue. These findings were confirmed by mass spectrometry, except for a few samples that were either too "dirty" or in which the NA concentration was too small for confirmation to be easily accomplished. Analysis of the data indicated that 69% NDMA and 74% NPYR formed in the polar fraction of adipose tissue. Less than 5% of either NA was found in the residual tissue, and less than 1% in the nonpolar fraction. Clearly, the principal NA precursors are contained in polar fraction I of the adipose tissue.

For further isolation of the NA precursors, a concentrated solution of the aqueous polar extract was subjected to gel filtration chromatography. Several Sephadex gels, including G-75, G-25, G-15, and G-10, were tested. The best separation was achieved with 40–120μ Sephadex G-15. Initially, Sephadex separations were carried out at room temperature, but breakdown of the dextran gel by the extract occurred. This was eliminated by carrying out the separation in a cold room at 4°C. Separation of the polar fraction with an ammonium acetate solution resulted in three major UV (254 nm) absorbing peaks, labelled B,

Table 1—Nitrosodimethylamine in adipose tissue and its fractions

Sample no.	Nitrosodimethylamine, ppb: Adipose tissue	Polar fraction (I)	Nonpolar fraction (II)	Residue (III)
1	160[a]	122[a]	2	4[a]
2	79[a]	62[a]	4	26[a]
3	61[a]	24	N.D.[b]	4
4	302[a]	144[a]	N.D.	9
5	205[a]	123[a]	N.D.	N.D.
6	148[a]	103[a]	N.D.	7
7	83[a]	72[a]	N.D.	4
8	97[a]	85	2	8[a]
9	103[a]	79[a]	3	9
10	213[a]	145[a]	N.D.	N.D.
11	125[a]	121	N.D.	4
Mean ± S.D.	143.3 ± 69.2	98.2 ± 35.7	1 ± 1.4	6.8 ± 6.8

[a] Confirmed by mass spectrometry.
[b] N.D. = None detected, <0.1 ppb.

Table 2—Nitrosopyrrolidine in adipose tissue and its fractions

Sample no.	Nitrosopyrrolidine, ppb: Adipose tissue	Polar fraction (I)	Nonpolar fraction (II)	Residue (III)
1	300[a]	242[a]	N.D.[b]	6[a]
2	132[a]	111[a]	2	18[a]
3	82[a]	23[a]	N.D.	N.D.
4	40[a]	28[a]	N.D.	11
5	136[a]	125[a]	4	N.D.
6	280[a]	218	N.D.	10
7	44	24	N.D.	N.D.
8	161[a]	125[a]	2	6
9	155[a]	127[a]	N.D.	2
10	306[a]	193[a]	13	11
11	138[a]	97[a]	N.D.	N.D.
Mean ± S.D.	161.3 ± 90.9	119.3 ± 72.5	1.9 ± 3.7	5.8 ± 5.8

[a] Confirmed by mass spectrometry.
[b] N.D. = None detected < 0.3 ppb.

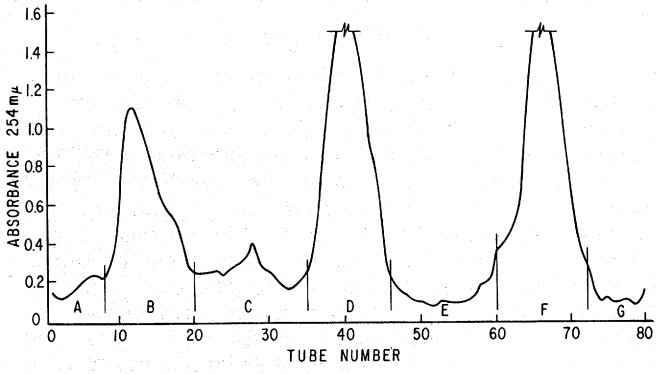

Fig. 2—Gel permeation chromatograph and UV absorption of aqueous polar fraction from adipose tissue on Sephadex G-15.

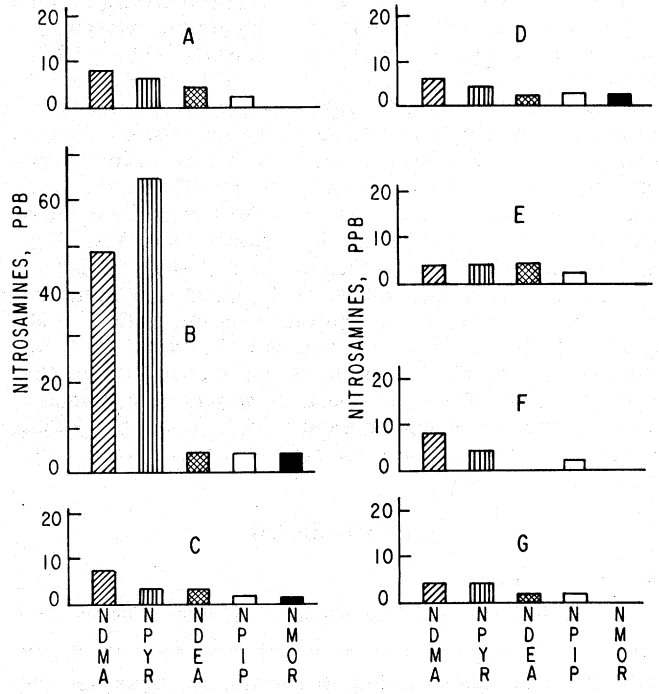

Fig. 3—Volatile nitrosamines in Sephadex G-15 subfractions of polar fraction from pork belly adipose tissue.

D, F, and several minor peaks (Fig. 2). From the shape of some of the chromatographic peaks, however, several compounds appeared to be present under each peak. Individual fractions from several runs were combined and lyophilized. The freeze-dried material was reconstituted in 2 ml H_2O and heated with 50 mg $NaNO_2$ and 25 g hydrogenated vegetable oil to yield NAs shown in Figure 3. The first major UV absorbing fraction, B, contained the highest concentration of NA precursors. Collating data from several similar experiments yielded mean NA values in subfraction B of 48 ppb NDMA, 64 ppb NPYR, and 4 ppb each of N-

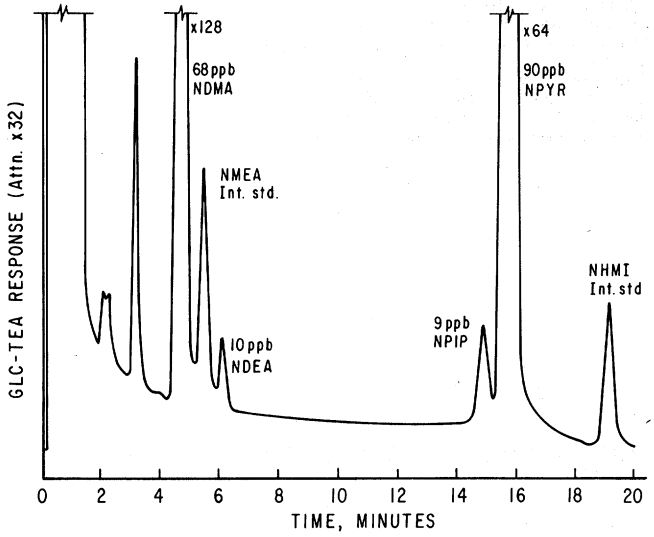

Fig. 4—GLC-TEA Chromatogram of nitrosamines formed fro polar subfraction B.

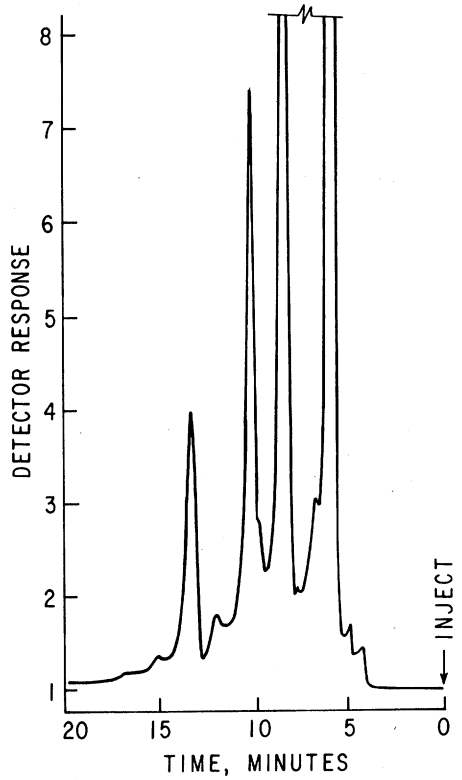

Fig. 5—HPLC Chromatogram of polar subfraction B on Bondapak μC_{18}.

nitrosodiethylamine (NDEA), N-nitrosopiperidine (NPIP), and N-nitrosomorpholine (NMOR). NDEA and NPIP have been detected previously in bacon but are not normally observed in the United States. NMOR has not been reported previously in fried bacon although the essential precursor appears to be present. All other subfractions contained substantially lower concentrations of the volatile NAs: 4–8 ppb NDMA, 4–6, ppb NPYR, 2–4 NDEA, and 2–4 ppb NPIP. NMOR, 2–4 ppb, was observed only in subfractions B, C, D. A typical GLC-TEA chromatogram of subfraction B is shown in Figure 4. N-nitrosomethylamine (NMEA) and N-nitrosohexamethylene-imine (NHMI) were added as internal standards for analytical purposes.

Since the major NDMA and NPYR precursors were found in subfraction B, further separation was carried out by reverse-phase HPLC with a μC_{18} column. A typical chromatogram contains four major and a number of smaller peaks (Fig. 5). For further characterization, subfraction B was chromatographed on two Waters Protein Columns I-125 (7.8 mm x 30 cm) with 50 mM Na_2HPO_4 buffer at pH 7.2. The resulting separation indicated that the compounds in the fraction were of a molecular weight range of 1,000–2,000. This agrees with a molecular weight estimation of 600–1,200, determined by gel filtration chromatography on Sephadex G-15, with lactose with NADPH as molecular weight standards. The major constituents of subfraction B separated by TLC on Silica Gel G plates (isopropanol/NH_4OH–70/30) were indicated ninhydrin positive. There was no evidence for the presence of free proline in this fraction prior to being heated. Thus it appears that one or more compounds, possibly peptides, contained in pork belly adipose tissue are responsible for NPYR formation in bacon. This is contrary to the findings of other researchers whose studies on precursors have centered primarily on nitrosated or non-nitrosated proline, and to a lesser extent on hydroxyproline, pyrrolidine, and putrescine in model systems (Bills et al., 1973; Huxel et al., 1974). Other investigators have worked with green pork bellies and bacons to confirm the role of the above amino acids or amines as NPYR precursors. Mottram et al. (1976) and Spinelli-Gugger et al. (1980) found that free proline in the adipose or collagenous tissue present in bacon could not account for the amount of NPYR found in fried bacon. Other factors, including physiochemical conditions, present in adipose tissue were thought to account for NPYR formation (Patterson et al., 1976).

The possibility that adipose tissue may contain constituents which may catalyze nitrosamine formation cannot be ruled out, as evidenced by effects due to nitrite reacting with unsaturated fatty acids (Walters et al., 1979; Goutefongea et al., 1977), thiols, and fatty acid peroxide (Coleman, 1978). Possibly several factors acting synergistically, and all possibly present in the aqueous extract, are responsible for the formation of NAs in bacon. Additional work is being carried out to furthe isolate and characterize the individual precursor constituents present in the pork belly adipose tissue.

REFERENCES

Bills, D.D., Hildrum, K.I., Scanlan, R.A., and Libbey, L.M. 1973. Potential precursors of N-nitrosopyrrolidine in bacon and other fried foods. J. Agric. Food Chem. 21: 876.

Coleman, M.H. 1978. A model system for the formation of N-nitroso pyrrolidine in grilled or fried bacon. J. Food Technol. 13: 55.

Enders, F. and Ceh, L. 1971. Conditions and chemical reaction mechanisms by which nitrosamines may be formed in biological products with reference to their possible occurrence in food products. Z. Lebensm. Unters. Forsch. 145: 133.

Fazio, T., Howard, J.W., and White, R. 1972. In Proceedings Conf. "N-nitroso Compounds—Analysis and Formation," Ed. Bogovski, P., Preussman, R., and Walker, E.A. IARC Scient. Publ., No. 3, p. 16. International Agency for Research on Cancer, Lyon.

Fiddler, W., Pensabene, J.W., Fagan, J.C., Thorne, E.J., Piotrowski, E.G., and Wasserman, A.E. 1974. The role of lean and adipose

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tissue on the formation of nitrosopyrrolidine in fried bacon. J. Food Sci. 39: 1070.
Goutefongea, R., Cassens, R.G., and Wooldord, G. 1977. Distribution of sodium nitrite in adipose tissue during curing. J. Food Sci. 42: 1637.
Gray, E.T., Scanlan, R.A., and Libbey, L.M. 1974. Formation of N. nitrosopyrrolidine from pyrrolidine ring containing compounds at elevated temperatures. J. Agric. Food Chem. 22: 698.
Lakritz, L. and Kimoto, W. 1980. N-nitrosamines—contaminants in blood collection tubes. Food Cosmet. Toxicol. 18: 77.
Magee, P.N. 1971. Toxicity of nitrosamines: Their possible human health hazards. Food Cosmet. Toxicol. 9: 207.
Mottram, D.S., Edwards, R.A., and Patterson, R.L.S. 1976. The distribution of N-nitrosopyrrolidine between lean, fat, and vapour in frying bacon. 22nd European Meat Research Workers Conference. Malmo, Sweden, F11: 3.
Mottram, D.S., Patterson, R.L.S., Edwards, R.A., and Gough, T.A. 1977. The preferential formation of volatile N-nitrosamines in the fat of fried bacon. J. Sci. Food Agric. 28: 1025.
Nakamura, M., Baba, N., Nakaoka, T., and Wada, Y. 1976. Pathways of formation of N-nitrosopyrrolidine in fried bacon. J. Food Sci. 41: 874.
Patterson, R.L.S., Taylor, A.A., Mottram, D.S., and Gough, T.A. 1976. Localised occurrence of N-nitrosopyrrolidine in fried bacon. J. Sci. Food Agric. 27: 257.
Pensabene, J.W., Feinberg, J.I., Dooley, C.J., Phillips, J.G., and Fiddler, W. 1979. Effect of pork belly composition and nitrite level on nitrosamine formation in fried bacon. J. Agric. Food Chem. 27: 842.
Pensabene, J.W., Fiddler, W., Gates, R.A., Fagan, J.C., and Wasserman, A.E. 1974. Effect of frying and other cooking conditions on nitrosopyrrolidine formation in bacon. J. Food Sci. 39: 314.
Spinelli-Gugger, A.M., Lakritz, L., and Wasserman, A.E. 1980. Effect of processing on the amino acid composition and nitrosamine formation in pork belly adipose tissue. J. Agric Food Chem. 28: 424.
Walters, C.L., Hart, R.J., and Perse, S. 1979. The possible role of lipid pseudonitrosites in nitrosamine formation in fried bacon. Z. Lebensm. Unters. Forsch. 168: 177.
White, R.H., Habery, D.C., Roseboro, E.L. and Fazio, T. 1974. Isolation of volatile N-nitrosamines in edible vegetable oils and cooked bacon fat. J. Assoc. Off. Anal. Chem. 57: 1380.
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NOTE: Precautions should be exercised in the handling of nitrosamines since they are potential carcinogens.

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